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54 Membranes with bound oligonucleotides and peptides.

57 A method is provided for synthesizing oligonucleotides and peptides directly onto a membrane. The method provides a means for generating membrane affinity supports. A modified membrane for the method of direct synthesis is also provided.

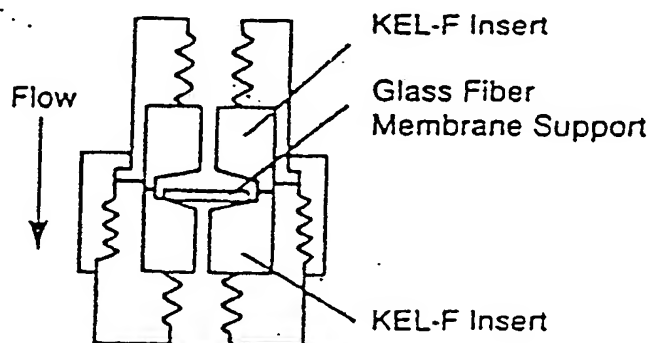


FIGURE 1

EP 0 305 929 A2

sequences on the antibody to react strongly and specifically with the antigen (H.M. Geysen et al., *Proc Nat'l. Acad. Sci. USA*, vol. 82, page 3998, (1984)). Polyethylene pegs are only useful for very specific purposes and suffer from the extreme low loading of immobilized biomolecules due to the non-porous structure. In a description of a process for the simultaneous chemical synthesis of several oligonucleotides paper discs have been used (DE 3301833 and EP 114599). This material cannot be recommended to serve as affinity support, because the material apparently does not allow to use the state-of-the-art phosphoramidite chemistry for the construction of long oligonucleotides with more than one hundred nucleotide units in the sequence (N.D. Sinha et al, *Nucleic Acids Res.*, 12:4539, (1984)). With the phosphate triester method (see e.g. M. Gait as cited above) only relatively short oligonucleotides (in the range of twenty nucleotide units containing sequences) can be obtained with the paper disc method. Moreover, after a few synthetic cycles employing the necessary treatment with different reagents and washing steps the paper gets very fragile and loses its mechanical stability. No peptides have been synthesized so far on paper; it is very probable that due to the harsh conditions necessary to synthesize peptides the cellulose matrix will be disrupted. Thus, affinity supports cannot be obtained by virtue of chemical synthesis of oligonucleotides or peptides onto paper as solid support.

Nucleic acids and peptides or proteins have been immobilized onto beaded and flat polymeric supports either by adsorption or by non-specific covalent linkage. To mediate an efficient and specific interaction using hybridization or affinity techniques between the soluble and immobilized biomolecules, a specific covalent attachment of the biomolecule involving only one terminal function would be optimal. This would make available the whole sequence of the immobilized biomolecule to interact with the complementary molecule in solution. Adsorption or non-specific covalent binding, however, involves several functions in the biomolecule, which are then rendered unavailable for the desired intermolecular interaction. Adsorption has furthermore the disadvantage that some of the immobilized biomolecules can be washed out (desorbed) during the hybridization or affinity process. This has to be particularly considered if the affinity support should be reused several times.

Whereas the terminus specific covalent attachment of oligonucleotides or peptides onto solid supports using the stepwise synthetic approach has been performed using beaded supports or paper discs (in the case of oligonucleotides) or beaded supports and polyethylene pegs (in the case of oligopeptides) no synthesis of these biopolymers has been reported employing membrane-type supports.

A membrane being flat and highly porous, mechanically stable material, would be suitable for upscaling purposes and reused several times. Furthermore, the support should be chemically stable under the conditions of oligonucleotide and peptide synthesis and should not show non-specific binding of either nucleic acids or proteins as this would give rise to a sensitivity-reducing background interaction. The development of an affinity support which fulfills these different requirements is not a trivial task. Whether the direct chemical synthesis of oligonucleotides or peptides is possible on such an insoluble support can also not be predicted. As mentioned, paper could only serve as a support for solid phase oligonucleotide synthesis when the phosphotriester approach was employed; for reasons which are still unclear, the much more efficient and state-of-the-art phosphoramidite chemistry which is very successfully used on porous glass beads did not work on paper.

#### Summary of the Invention

This invention pertains to a method of synthesizing oligonucleotides (DNA and/or RNA fragments) or peptides covalently and specifically linked to membranes. The invention also pertains to modified membranes for synthesis of oligonucleotides and peptides and to membranes having oligonucleotides or peptides attached thereto by a terminal specific attachment (the biopolymer is covalently bound at one of its termini).

According to the method of this invention a modified membrane is employed which is represented by the formula:



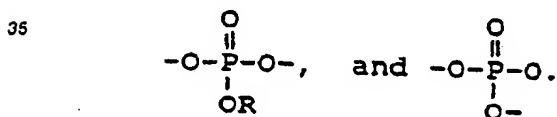
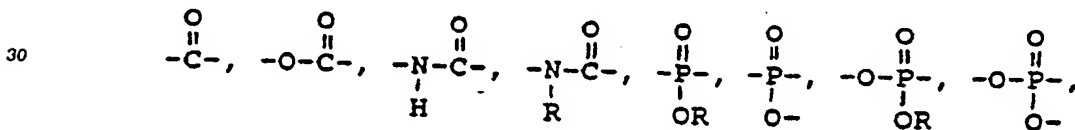
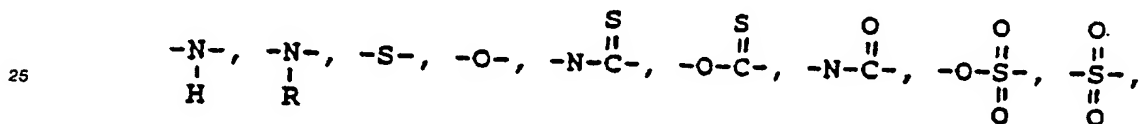
wherein P represents a polymeric membrane support linked to a protected nucleoside or amino acid  $S^W$ , where W represents protecting groups, through a linker Y-N-Z, where N represents a spacer group and Y and Z represent the same or different functional groups, the linker being bound to the membrane through a functional group X on the membrane.

by reduction or Grignard reaction. Other types of polymers can generate free functional groups by partial hydrolytic reactions. Polyvinylidene difluoride (PVDF) can generate functional groups (double bonds) by dehydrohalogenation.

C. Chemically inert polymers such as polysulfones, polytetrafluoroethylene (Teflon™), polyethylene, polypropylene, polyvinylidene difluoride (PVDF) can be activated by radiation e.g. with high energy UV or Cobalt-60 and the generated ions or radicals used for grafting onto the surface of the polymer, chains containing monomers with functional groups according to A and/or B.

D. Chemically inert polymers such as polysulfones, polytetrafluoroethylene (Teflon™), polyethylene, polypropylene, polyvinylidene difluoride (PVDF) can be coated with copolymers, which already do contain free functional groups (A) or easily transformed to generate functional groups by using conventional chemical or physico-chemical processes (B,C). Another subtype could be obtained by crosslinking e.g. polyvinylalcohol on the surface of the aforementioned polymers, generating diradicals by reacting the cis-diol structure with Cer(IV)nitrate and use the radicals to start a grafting process involving monomers according to A and/or B.

Y-N-Z is a bifunctional group, in which Y reacts with the functional group X on the polymer and mediates via Z linkage to the first synthetic building block either a suitably protected nucleoside or amino acid derivative. N is a spacer group. Any suitable spacer group can be used. Substituted or unsubstituted alkyl, aryl, aryl alkyl groups are suitable. For example, N can be a variable spacer consisting of n CH<sub>2</sub> groups, n varying between 1 and 20. Spacing can also be accomplished by chains such as oligoglycine or -NH-(CH<sub>2</sub>)<sub>m</sub>-NHCO-(CH<sub>2</sub>)<sub>m</sub>-CO, m being, for example, 1 to 6. Y and Z can be the same or different and selected from a variety of standard functional groups, such as:



wherein R is alkyl, aryl, aralkyl, or cycloalkyl.

S represents a suitably protected first building block anchored to the membrane support P such as a nucleoside or an amino acid. The nucleoside is represented by the formula:



wherein W'' is H or a suitable hydroxy protecting group such as trityl groups, acyl groups or silyl groups. B is a nucleoside base such as adenine, guanine, cytosine, thymine, uracil or analogs of these bases. For example W' can represent a base-labile acyl group generally used for protection of exocyclic amino groups on the heterocyclic nucleoside bases. The nucleoside is generally attached to the membrane via the 3' position but can be attached at the 5' position. When attached to the membrane that the 3' position, the 5' carbon can contain a protected hydroxy group. Preferred protecting groups for the 5' hydroxy group are

b) coupling a 5'-DMT- and N-protected 3' phosphoramidite after activation with a suitable activator such as tetrazole or 4-nitrophenyltetrazole to the free 5'-OH group of the membrane-bound deoxynucleoside;

c) capping non-reacted 5'-OH groups of the immobilized deoxynucleoside (or oligonucleotide) with reagents such as acetic acid anhydride/N,N-dimethylaminopyridine, thereby reducing the occurrence of failure sequences; and

d) oxidizing the trivalent phosphite triester bond with reagents such as iodine/ 2,6-lutidine/water to the pentavalent phosphate triester bond.

Between the different reaction steps of the elongation cycle appropriate washing steps are employed. Steps a) through d) are repeated using in step b) the correct building block until the desired oligonucleotide sequence is generated.

In the preferred mode the beta-cyanoethyl phosphoramidite chemistry is employed. See Sinha et al., *Nucleic Acids Res.* 12:4539 (1984). See also, U.S. Patent Application No. 752,178 filed June 18, 1985, the teachings of which are incorporated by reference herein. This technique comprises coupling a nucleoside beta-cyanoethyl protected phosphoramidite to the membrane-bound nucleoside to produce a membrane-bound nucleoside-nucleotide having a phosphite triester, oxidizing the phosphite triester to form a phosphate triester linkage and sequentially coupling additional nucleoside beta-cyanoethyl protected phosphoramidites to the membrane-bound nucleoside-nucleotide and after each coupling step, oxidizing the resulting phosphite triester linkage to produce a membrane-bound polynucleotide.

To use the oligonucleotide-membrane as an affinity support for hybridization experiments the N-protecting groups of the nucleoside bases must be removed to enable Watson-Crick base pairing. Usually the phosphate protecting group (e.g. beta-cyanoethyl) is also removed to generate the naturally occurring internucleotidic linkage (phosphodiester bond). It may be of advantage, however, to keep the phosphate protecting groups. In some cases (e.g. when synthesizing the unnatural oligomethyl-phosphonate diesters) the internucleotidic linkage remains 'protected'. The synthesized oligonucleotide can also be cleaved from the membrane. It depends on the selection of X-Y-N-Z functions (formula 1) and the choice of phosphate and N-protecting groups (and 2'-OH protecting groups in the case of oligoribonucleotide synthesis) whether the oligonucleotide remains linked to the membrane (as necessary if the membrane is to serve as an affinity support) or is cleaved off the carrier during or after deprotection. It is an advantageous feature of this invention that out of the large selection of protecting groups known in the art a selection can be made which allows (by employing different sets of conditions) the oligonucleotide either to be cleaved off the membrane or to be left on the membrane after appropriate deprotection to allow hybridization on the membrane. In some cases a sequence specific optimization process should be worked out to generate high yields and a homogeneous product; for this optimization process it is necessary to identify and to characterize the oligomeric product. Once the optimal conditions have been worked out the affinity support is generated by removing only those protecting groups necessary to allow the affinity process to take place.

#### Peptide synthesis:

In state-of-the-art peptide synthesis, prior to anchoring the first amino acid building block to the solid support, the unnatural amino acid norleucine and a special linker molecule are attached to the solid support. The norleucine residue acts as an internal standard for the subsequent amino acid analysis of the synthesized oligopeptide; the linker molecule provides a benzyl alcohol function to esterify the first amino acid building block to the solid support. Various linker molecules are in use, which differ in reactivity of the ester linkage (see e.g., R.L. Sheppard & B.J. Williams, *Int. J. Peptide & Protein Res.*, vol. 20, page 451, 1982).

Scheme III describes the preparation of Immobilon affinity membrane IAM 4 (n=2) for the synthesis of peptides. First 4 is reacted with the active, pentafluorophenyl (Pfp) ester of norleucine 7, which is protected at the primary amino function with the fluorenylmethoxycarbonyl (Fmoc) group to furnish 8. Remaining amino groups of 4 are capped with acetic acid anhydride (step a of scheme III) and thereafter the Fmoc group is removed by treatment with 20% piperidine in N,N-dimethylformamide (step b of scheme III) resulting in the formation of 9. The primary amino group of 9 is then reacted with the pentafluorophenyl ester of the linker molecule 10 yielding the membrane derivative 11 ready for esterification to the first amino acid building block via its carboxyl terminus. The selection of p-hydroxymethylphenoxyacetic acid as linkage agent provides for an acid labile linkage to the synthesized peptide sequence. The first amino acid building block 12 is coupled to 11 via its symmetrical anhydride in the presence of N,N-dimethylaminopyridine as catalyst to generate the membrane derivative 13, which now carries a covalently and specifically attached

mL of pyridine/acetic acid anhydride, 3/1 (v/v), for 2.0 hours at 20° C. The membrane was washed with methanol and dried. A small portion (5 mg) of the support 6 (scheme II) was assayed for the presence of the dimethoxytrityl group ( $\epsilon_{498} = 74,500$  in 70% perchloric acid/ethanol, 1/1 (v/v)). The assay indicated 0.032 mmol of nucleoside bound per gram of dry membrane 6, 80% yield.

## EXAMPLE 3

## Synthesis of d(T-C-C-C-A-G-T-C-A-C-G-A-C-G-T-C)

A 0.8 cm<sup>2</sup> disc of the membrane 6 (B = cytosine, W = benzoyl) was placed in a specifically designed holder (figure 1) and fitted into a MilliGen 6500 automated DNA synthesizer. The above sequence was assembled automatically by using  $\beta$ -cyanoethylphosphoramidites (N.D. Sinha et al., as cited above) and a standard synthetic protocol. Following the last addition cycle the membrane disc was treated in a sealed tube with 0.3 mL of conc. aqueous ammonia for 12 hours at 55 C. The ammoniacal solution was concentrated and chromatographed by reverse phase hplc. The hplc chromatogram is shown in figure 2. The product peak was analyzed by polyacrylamide gelelectrophoresis (as described in N.D. Sinha et al.) The result is shown in Figure 3a. The material in the main band was subjected to sequence analysis using the Maxam & Gilbert procedure (as described in N.D. Sinha et al, is cited above). The result is shown in figure 3b demonstrating the correctness of the synthesized hexadecamer sequence.

## EXAMPLE 4

Attachment of norleucine to IAM 4

Immobilon affinity membrane 4 (n = 2, scheme I), 3.20 g (0.349 mmol of amino groups), was reacted with N-Fmoc-Nle-O-Pfp (6.0 mmol) in the presence of 1-hydroxybenzotriazole (6.0 mmole) in 20 mL of dry DMF for 2.0 hours at room temperature. The support was washed with methanol, dried, and then treated for an additional 2.0 hours at room temperature, with 40 mL pyridine/acetic acid anhydride, 3/1 (v/v). The acylation reaction was terminated by washing the membrane with methanol. The amount of incorporated norleucine was 0.093 mmol/g membrane as determined by quantitation of the fluorenylmethoxycarbonyl moiety. The assay is performed by carefully weighing in 5.0 mg of the membrane 8 (scheme III) and treatment with 0.4 mL of a mixture of piperidine and 0.4 mL dichloromethane for 30 minutes at room temperature. The solution was diluted to 10.0 mL with dichloromethane and the absorbance at 301 nm determined ( $\epsilon_{301} = 7,800$  for N-fluorenylmethyl piperidine in dichloromethane).

## EXAMPLE 5:

Attachment of the linker moiety 10 to H-Nle-IAM 9

Membrane 9, 3.2g (0.30 mmol of amino groups) was placed in a dish containing 50 ml of 20% piperidine in dimethylformamide. After 10 minutes at 20° C, the membrane was washed 10 times with small portions of dry dimethylformamide. The wet material was then treated with 6.0 mmol of 4-hydroxymethyl

## Synthesis of Oligonucleotides on Polypropylene Membranes

5 A polypropylene membrane (0.180g) grafted with polyethoxyethyl acrylate was treated with 2.0 mmol of O-dimethoxy trityl aminoethanol in 2.0 ml of DMF for 19 hrs at 80° C. The membrane was washed with methanol and dried. A small portion of the material was assayed for the presence of the dimethoxytrityl group (see example 2). The assay revealed the polymer contained 0.0022 mmol of protected alcohol functional group per gram of polymer.

10 A 0.8 cm<sup>2</sup> disc of the membrane was placed in the specially designed holder of figure 1 fitted in a MilliGen 6500 DNA Synthesizer. The synthesis of

d(T-C-C-C-A-G-T-C-G-A-C-G-T)

was conducted using a standard phosphoramidite synthesis protocol (Sinha et al. supra). At the conclusion of the synthesis, the disc was treated with 0.3 ml of concentrated aqueous ammonia for 12 hours at 55° C. 15 Acid hydrolysis of the 5' terminal dimethoxytrityl group indicated 0.0003 mmol of oligonucleotides per gram of dry membrane. This indicated an overall step-wise yield of 88%.

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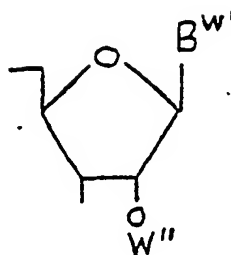
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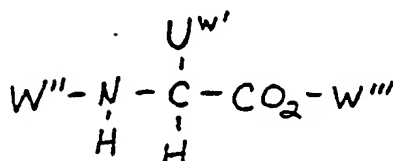
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wherein  $B^W$  is a protected nucleobase; and  $W''$  is H or a hydroxy protecting group.

18. A modified membrane of Claim 17 wherein  $W''$  is a trityl group, an acyl group or a silyl group.

19. A modified membrane of Claim 2 wherein S represents an amino acid of the formula:



wherein U represents an amino acid side chain;

$W'$  represents side chain protecting groups;

$W''$  represents a amino protecting group; and

$W'''$  represents a carboxy protecting group.

20. A modified membrane of Claim 19 wherein  $S^W$  is norleucine attached to the linker through its carboxyl group.

21. A modified membrane of Claim 20, wherein the primary amino group of the norleucine is protected with Fmoc fluoroenylmethoxycarbonyl.

22. A method of synthesizing an oligonucleotide comprising sequentially coupling nucleotide to a modified membrane of Claim 17.

23. A method of synthesizing a peptide comprising sequentially coupling amino acids to a modified membrane of Claim 19.

24. A method of synthesizing an oligonucleotide comprising the steps of:

a. providing a modified membrane represented by the formula:



wherein P is a polymeric membrane;

X is a functional group on the membrane;

Y-N-Z is a linker wherein N is a spacer molecule and Y and Z are the same or different functional groups, the linker being bound to the membrane through the functional group X; and

$S^W$  is a protected nucleoside,  $S^W$  being bound to the linker through the functional group Z of the linker;

b. coupling a protected nucleoside phosphoramidite to the nucleoside  $S^W$  to produce a membrane-bound nucleoside-nucleotide having a phosphite triester linkage;

c. oxidizing the phosphite triester to form a phosphate triester linkage; and

d. sequentially coupling additional protected nucleoside phosphoramidite to the membrane-bound nucleoside-nucleotide, and after each coupling step, oxidizing the resulting phosphite triester linkage to a phosphate triester to produce a membrane-bound polynucleotide.

25. A method of Claim 24, wherein the nucleoside phosphoramidite contains a beta-cyanoethyl protected phosphate.

26. A method of Claim 24, further comprising removing the protecting groups from the membrane bound polynucleotide.

27. A method of Claim 26, wherein the synthesized polynucleotide is cleaved from the membrane.

28. A membrane-bound polynucleotide produced by the method of Claim 24.

29. A method of synthesizing a peptide, comprising the steps of:

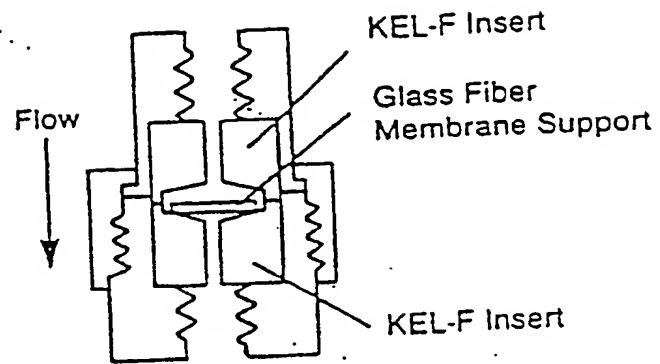
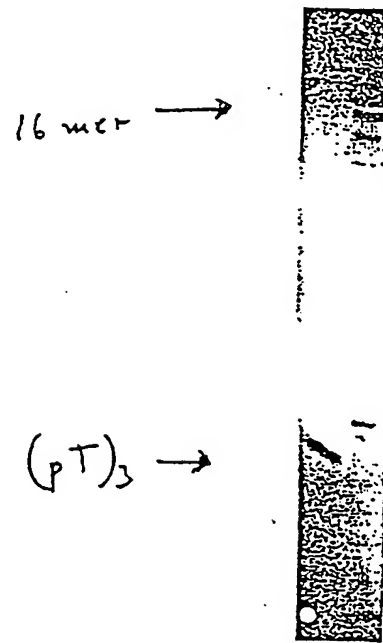


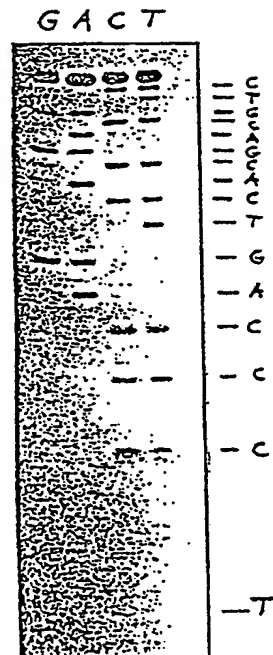
FIGURE 1



FIGURE 3



a



b

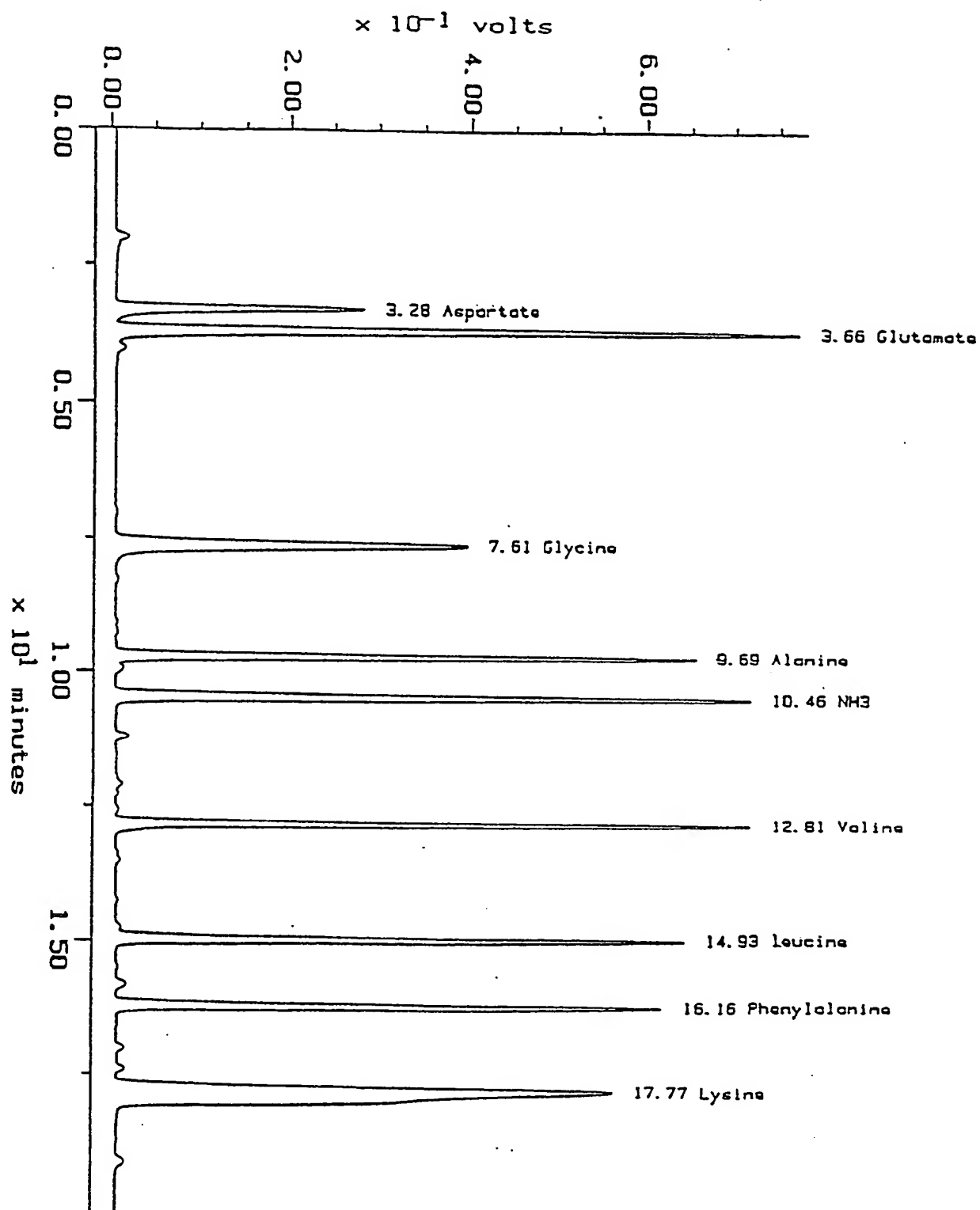


FIGURE 5